Staphylococci Isolated from Raw Milk of Yak and Cattle in Mongolia

Studies on the occurrence, characterization, detection of enterotoxin and antimicrobial susceptibility profile of the isolates

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Uppsala 2006

The present thesis is a partial fulfilment of the requirements for the Master of Science Degree for International Students (MSc) in Veterinary Medicine, at the Swedish University of Agricultural Sciences (SLU), in the field of Veterinary Microbiology.

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Print: SLU Service/Repro, Uppsala 2006

To my family

My Motherland

The crystal rivers of sacred Kherlen, Onon and Tuul
Brooks, streams, and springs that bring health to all my people,
The blue lakes of Khuvsgul, Uvs and Buir-deep and wide,
Rivers and lakes where people and cattle quench their thirst;
This is my native land, the lovely country-my Mongolia.

The land of pure grasses waving in the breeze,
The land of open planning full of fantastic mirages,
Firm rocks and out-of -reach places where good man used to meet,
And the ancient ovoos-the standing stones to gods and ancestors;
This is my native land, the lovely country – my Mongolia

Land where in winter all is covered with snow and ice,
And the grassestwinkle like glass and crystal,
Land where in summer all is carpet of flowers,
And full of songbirds from the distant lands to the south;
This is my native land, the lovely country-my Mongolia.

D. Natsagdorf

Abstract

Tsegmed, U. 2005. *Staphylococci isolated from raw milk of yak and cattle in Mongolia. Studies on the occurrence, characterization, detection of enterotoxin and antimicrobial susceptibility profile of the isolates.* Master's thesis.
ISSN 1403-2201

Occurrence, characterization, detection of enterotoxin and antimicrobial susceptibility profile in staphylococci isolated from yak and cattle in Mongolia were investigated. Staphylococci were isolated from 72 (74%) of the 97 raw milk samples investigated. Of the samples containing staphylococci, 69% (50/72) were from yak, whereas 31% (22/72) were from cattle. Of the samples containing staphylococci, S. aureus was detected in 14% (7/50) of yak milk samples and in 68% (15/22) of cattle samples. Staphylococcal enterotoxin C (SEC) was detected in 19% (5/26) of the S. aureus strains investigated. Three of the enterotoxigenic strains were from yak and two from cattle. None of the *S. aureus* strains tested produced SEA, SEB, or SED.

The MICs of 12 antimicrobial agents for 45 isolates of *Staphylococcus* spp. from yak and cattle were determined. Broth microdilution was used for the susceptibility testing and because of high oxacillin MICs all isolates were also subjected to oxacillin agar screening and PCR for the mecA gene. Nitrocefin test was used to determine β-lactamase production. The proportion of resistance to β-lactamse based on β-lactamase production was high (37-84%). However, no mecA gene was detected. Resistance to erythromycin, clindamycin, chloramphenicol, tetracycline, trimethoprim and fusidic acid was recorded among isolates from both yak and cattle. Cephalothin resistance was found only among coagulase-negative staphylococci from yak.

Keywords: staphylococci, occurrence, enterotoxin, antimicrobial susceptibility, milk, yak, cattle, Mongolia

Contents

Introduction

Livestock production in Mongolia is crucial for the life of the people and for the national country's economy. Cattle production is of particular importance, with a population of 1.8 million heads, of which 460.000 are yak. Cattle as a whole account for 80% of milk production and 40% of meat products.

The yak (*Poephagus grunniens* or *Bos grunniens*) is one of the most remarkable domestic animals in the world, adapted to living in high mountain terrain in harsh conditions (Roginski et al., 2003; Magash 2003). The yak is inherently associated with the culture, religion and social life of the Mongolians. Yak is kept in 13 of 22 provinces and 70% of yak herds are concentrated in the Hangai and Hovsgol mountains. Yak grazing pastures lie on plateaus between 1500 and 4000 m above sea level (Tomorjav, 1989). Yak is used for production of milk, meat, hair/wool, leather, and manure for heating, as well as for transportation of both people and goods. Dairy products mainly milk, cheese, and yoghurt are the primary ingredients of the Mongolian diet. Yak milk is consumed fresh, or preserved in different ways, such as cheese, melted butter, soured milk, yoghurt and flavoured curds.

Clinically, the most important genus of the *Micrococcaceae* family is *Staphylococcus*. Forty species and 24 subspecies of the genus *Staphylococcus* are described in the current version of the List of Prokaryotic Names with Standing in Nomenclature, LPSN (Euzéby, 2005). The *Staphylococcus* genus is classified into two major groups: coagulase-negative staphylococci (CNS) and coagulase-positive staphylococci (CPS). CNS, comprising the majority of species, are considered to be saprophytic or, rarely, pathogenic (Kloos and Schleifer, 1975), but the importance of CNS is increasing in the hospital environment and the veterinary medicine (Bautista et al., 1988; Kloos and Bannerman, 1994; Thorberg and Brändström, 2000). Recently, eleven staphylococcal species have been sequenced: *S. aureus*, *S. epidermidis, S. saprophyticus, S. haemolyticus, S. hominis, S. cohnii, S. auricularis, S. capitis, S. simulans, S. warneri and S. lugdunensis* (Martineau et al., 2001).

Staphylococcus aureus is a leading cause of community-acquired infections in humans and a cause of mastitis and skin diseases in milk producing animals (Akineden et al., 2001; Nagase et al., 2002; Klotz et al., 2003; Lim et al., 2004; Katsuda et al., 2005). Moreover, among food-borne intoxications, *S. aureus* is a major cause of gastroenteritis resulting from consumption of contaminated food products (Wieneke et al., 1993; Le Loir et al., 2003; Bennett, 2005). Outbreaks of *S. aureus* food poisoning have been caused by the consumption of dairy products, including raw milk (Evenson et al., 1988; Carmo et al., 2004; Jörgensen et al., 2005), low-fat milk and dried skimmed milk (Asao et al., 2003) and cheeses (Rosec et al., 1997).

Over the past 50 years, staphylococci (especially *S. aureus*) have become resistant to various antimicrobial agents including the commonly used penicillin-related antibiotics. Methicillin-resistant *S. aureus* (MRSA) is a bacterium resistant to certain antibiotics such as oxacillin, methicillin and other beta lactams

(Chambers et al., 1997; Lee, 2003; Boyce et al., 2005). MRSA strains have become a major concern for hospital epidemics in many countries (Maple et al., 1989; Witte et al., 2001). On the other hand, reports of MRSA in animals have been infrequent so far (Seguin et al., 1999; Lee, 2003; van Duijkeren et al., 2004; Rich, 2005; Loeffler et al., 2005).

In this study, the occurrence, characterization, detection of enterotoxin, and the antibiotic susceptibility profile of staphylococci isolated from raw milk samples from yak and cattle in Mongolia is presented.

Study of literature

General characteristics of the organisms

Staphylococci are Gram-positive cocci, 0.5 to 1.5 µm in diameter, which occur singly and in pairs, tetrads, and form grape-like clusters. It was 1883 when Ogston introduced the name staphylococcus (staphyle= bunch of grapes). One year later, Rosenbach used the term in a taxonomic sense and provided the first description of the genus S*taphylococcus*.

Staphylococci are aerobic and facultative anaerobic, catalase-positive, oxidase-negative, non-motile, non-sporeforming and fermentative. Colonies appear smooth, raised, glistening, circular, entire. Single colonies can attain a size of 4-6 mm in diameter on non-selective media. Colony colour is variable, from grey or grey-white to orange (Carter et al., 1994; Roginski et al., 2003).

Natural habitats and other sources

Staphylococci are widespread in nature; their major habitats include the skin and mucous membranes, especially of the upper respiratory tract and digestive tract of humans and other animals. The organisms have been isolated sporadically from soil, air, water, sewage, plant surfaces and products, feeds, dairy products, and kitchen worktops for food preparation. The incidence in human carriers ranges from 4% to 60% (Carter et al., 1994; Biberstein and Hirsh 1999; Uemura et al., 2004).

Diseases caused by the genus *Staphylococcus*

Coagulase-negative staphylococci (CNS) most frequently causing diseases in humans are *S. epidermidis* (nosocomial pathogen), *S. saprophyticus* (urinary tract infections), *S. haemolyticus* (endocarditis, peritonitis, septicemia) (Martineau et al., 2000; Cunha Mde L et al., 2004).

Of the coagulase-positive staphylococci (CPS), *S. aureus* most frequently causes diseases in humans in various suppurative (pus-forming) infections. It causes superficial skin lesions such as boils, styes and furunculosis; more serious infections such as pneumonia, mastitis, and urinary tract infections; and deep-seated infections such as osteomyelitis and endocarditis *(*Jarvis and Martone 1992; Ellis et al*.,* 2003). *Staphylococcus aureus* is also a serious bacterial cause of food-borne infections. Staphylococcal food poisoning is one of the economically most serious food-borne diseases worldwide (Evenson et al., 1988; Bennett, 2005).

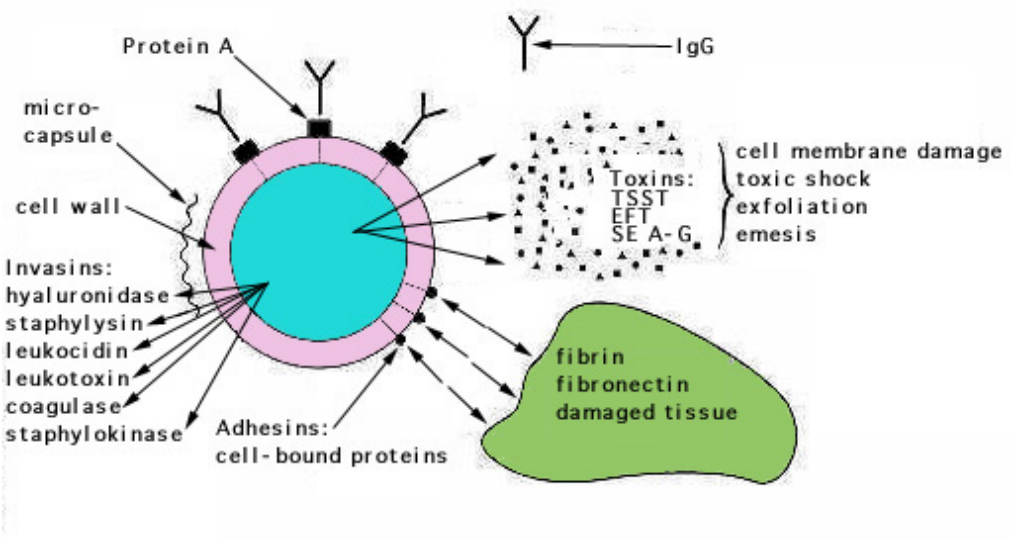
In animals, *S. aureus* can cause pustular inflammation of the skin and other organs, mastitis being the most serious (Garcia et al., 1980; Lee et al., 1998; Zschock et al., 2000; Nagase et al., 2002). *Staphylococcus aureus* is an important cause of mastitis in cattle, sheep and goats (Yazdankhah et al., 2001; Rodrigues da Silva et al., 2005). *Staphylococcus intermedius* causes pyoderma, staphylococcal pustular dermatitis, and otitis externa in dogs and cats. *Staphylococcus hyicus*

causes exudative epidermitis, septic polyarthritis in pigs and rare cases of mastitis in cattle (Biberstein and Hirsh 1999).

Virulence factors

S. aureus, the most intensively studied species of *Staphylococcus*, produces a variety of extracellular proteins, toxins, and enzymes (Altemeier et al., 1982; Balaban and Rasooly, 2000; McCormick et al., 2003; Fueyo et al., 2005; Todar, 2005).

Fig 1. Summary of virulence factors of *S. aureus*.
Diagram from http://www.textbookofbacteriology.net (Todar, 2005), with permission.



A. Surface antigens

- **Capsular polysaccharides** inhibit opsonization and phagocytosis; protect from leukocyte destruction
- **Teichoic acid** regulate cationic concentration in cell membrane; is receptor for bacteriophages
- **Protein A** binds immunoglobulins via the non-specific Fc receptor; inhibits opsonization and phagocytosis
- **Adhesins** the surface proteins that bind to matrix proteins such as fibronectin, fibrinogen (clumping factor), collagen, etc.

B. Extracellular proteins (membrane-damaging toxins)

Hemolysins

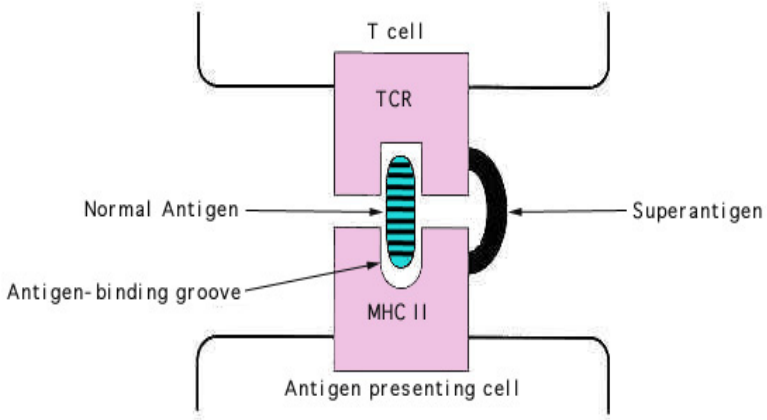
- **α-toxin,** the most potent membrane-damaging toxin, attacks rabbit erythrocytes and is responsible for the clear zone of hemolysis.

- **ß-toxin** is a sphingomyelinase that damages membranes rich in this lipid. The classical test for ß-toxin is lysis of sheep erythrocytes.
- **γ-toxin** is produced by two-component protein toxins that damage membranes of susceptible cells. The proteins are expressed separately but act together to damage membranes. The γ-toxin locus expresses three proteins: B and C components form a leukotoxin with poor hemolytic activity, whereas A and B components are hemolytic and weakly leukotoxic.
- **δ-toxin** is a very small peptide toxin produced by most strains of *S. aureus*. δ-toxin has an abroad hemolytic spectrum and is inhibited by phospholipids. The role of δ-toxin in disease is unknown.

Exotoxins–superantigens

- **Superantigens** that bind directly to class II major histocompatibility complex (MHC II) of antigen-presenting cells outside the normal antigen-binding groove and stimulate non-specific T-cell proliferation. Up to one in five T cells may be activated. Cytokines are released in large amounts, causing the symptoms of toxic shock (Balaban and Rasooly, 2000).

Fig 2. Superantigens and the non-specific stimulation of T cells.
Diagram from http://www.textbookofbacteriology.net (Todar, 2005), with permission.



- **Enterotoxins-superantigens**. Nine major antigenic types of *S. aureus* enterotoxins (SEs) have been identified and designated as SEA, SEB, SEC SED, SEE, SEG, SHE, SEI and SEJ (Borja and Bergdoll, 1967; Letertre et al., 2003; Blaiotta et al., 2004). The SEC can be subdivided into SEC1 SEC2 and SEC3, based on differences in minor epitopes (Bergdoll et al., 1965; Avena and Bergdoll, 1967). More recently, accumulating data have allowed of several new SE types by genome sequence analyses (Orwin et al., 2001; Omoe et al., 2003) and 20 distinct antigenic types of staphylococcal enterotoxins have been identified (Loncarevic et al., 2005). The symptoms of staphylococcal food poisoning are abdominal cramps, nausea, vomiting, and diarrhoea. Recovery usually occurs within 6 to 24 hours, depending on the

amount of food ingested, and the individual's general health (Le Loir et al., 2003).

- **Toxic shock syndrome toxin (TSST-1)-superantigen.** Toxic shock syndrome toxin is associated with strains that cause human toxic shock syndrome. TSST-1 not directly toxic to cells; it causes over-stimulation of T cells with efflux of lymphokines/ cytokines.
- **Exfoliative toxins (ET)-superantigen**
- Exfoliatin (epidermolytic toxin) causes a variety of dermatologic lesions known as staphylococcal scalded skin syndrome (SSSS). It cleaves the stratum granulosum of the epidermis.

Other exoproteins – not superantigens

- **Leukocidin** is highly leukotoxic but is non-hemolytic. Leukocidin is an important factor in necrotizing skin infections. It kills granulocytes and macrophages.
- **Coagulase** is an extracellular protein that binds to prothrombin in the host to form a complex called staphylothrombin. The protease activity characteristic of thrombin is activated in the complex, resulting in the conversion of fibrinogen to fibrin. This is the basis of the tube coagulase test, in which a clot forms in plasma.
- **Staphylokinase** (fibrinolysin): dissolves fibrin clots by promoting the conversion of plasminogen to the fibrinolytic enzyme plasmin.
- **Nuclease** (deoxyribonuclease) hydrolyses DNA.
- **Lipase** hydrolyses lipids.
- **Hyaluronidase** hydrolyses hyaluronic acid
- **Protease** hydrolyses proteins.

Identification

Staphylococci can be identified on the basis of colony morphology, production of coagulase, detection of hemolysins, thermostable deoxyribonuclease, by various enzyme activities, and aerobic acid production from certain carbohydrates. Commercial latex agglutination tests and API Staph system (bioMerieux) are examples of assays available for identification of staphylococci. Recently a real-time PCR assay was developed to identify common staphylococcal species (Brakstad et al., 1992; Klotz et al., 2003; Bennett, 2005; Pinto et al., 2005). There are also automated systems, such as Vitek and Baxter-MicroScan, which incubate inoculated trays or cards, read and interpret results, and with the aid of their programmed computer, determine the identity of organisms.

Antibiotic resistance

Staphylococcal disease has been a perennial problem in the hospital environment since the beginning of the antibiotic era. Widespread use of antibiotics is thought have engendered evolutionary changes in bacteria that allow them to survive these powerful drugs (De Oliveira et al., 2000; Gentilini et al., 2000; Erskine et al., 2002; Pitkälä et al., 2004). In the late 1950s and early 1960s, S. aureus was

responsible of considerable morbidity and mortality as nosocomial pathogen in hospitalised patients. Ninety percent of Staphylococcus strains are resistant to penicillin and penicillin-derived antibiotics. The next line of attack, methicillin, is becoming increasingly less effective; between 1975 and 1991, the prevalence of methicillin-resistant strains of S. aureus increased by 26% (Lieberman, P.B., Wootan, M.G. Protecting the Crown Jewels of Medicine. Available online at http://www.cspinet.org/reports/abiotic.htm).

For correct selection of antimicrobial agents for therapy it is extremely important that methicillin-resistant staphylococci be quickly correctly recognized. Recently, many molecular typing methods have been applied to the epidemiological analysis of *S. aureus*, especially of methicillin-resistant strains (MRSA) (Smyth et al., 2001; Lee et al., 2004).

Aims of the investigation

1. To investigate the occurrence of staphylococci in raw milk from yak and cattle in Mongolia.
2. To investigate enterotoxin production in *S. aureus* isolates.
3. To investigate the antimicrobial susceptibility of the isolates.

Summary of results presented in papers I and II

Paper I

In this paper, the occurrence of and enterotoxin production by *Staphylococcus aureus* isolated from raw milk from yak and cattle in Mongolia were investigated. Staphylococci were isolated from 72 (74%) of the 97 raw milk samples. Of the samples containing staphylococci, 69% (50/72) were from yak, whereas 31% (22/72) were from cattle. Of the samples containing staphylococci, *S. aureus* was detected in 14% (7/50) of yak milk samples and in 68% (15/22) of cattle samples. Staphylococcal enterotoxin C (SEC) was detected in 19% (5/26) of the *S. aureus* strains investigated. Three of the enterotoxigenic strains were from yak and two from cattle. None of the *S. aureus* strains tested produced SEA, SEB, or SED.

Paper II

The aim of this study was to determine the MICs of 12 antimicrobial agents for 45 isolates of *Staphylococcus* spp. from yak and cattle were determined. Broth microdilution was used for the susceptibility testing and because of high oxacillin MICs all isolates were also subjected to oxacillin agar screening and PCR for the *mecA* gene. Nitrocefin test was used to determine β-lactamase production. The proportion of resistance to β-lactase based on β-lactamase production was high (37-84%). However, no *mecA* gene was detected. Resistance to erythromycin, clindamycin, chloramphenicol, tetracycline, trimethoprim and fusidic acid was recorded among isolates from both yak and cattle. Cephalothin resistance was found only among coagulase-negative staphylococci from yak.

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Research report I

Occurrence of and Enterotoxin Production by *Staphylococcus aureus* Isolated from Raw Milk from Yak and Cattle in Mongolia

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Abstract

Staphylococcal food poisoning (SFP) is considered to be among the leading causes of food-borne illnesses worldwide and contaminated food of animal origin, such as milk and dairy products, is often responsible for the intoxication in humans. In this study we investigated the occurrence of and enterotoxin production by *Staphylococcus aureus* isolated from raw milk from yak and cattle in Mongolia. Staphylococci were isolated from 72 (74%) of the 97 raw milk samples. Of the samples containing staphylococci, 69% (50/72) were from yak, whereas, 31% (22/72) were from cattle. Of the samples containing staphylococci, *S. aureus* was detected in 14% (7/50) of yak and in 68% (15/22) of cattle milk samples. Staphylococcal enterotoxin C (SEC) was detected in 19% (5/26) of the *S. aureus* strains investigated. Three of the enterotoxigenic strains were from yak and two from cattle. None of the *S. aureus* strains tested produced SEA, SEB, or SED. This is the first report, to the authors' knowledge, of the occurrence of and enterotoxin production by *S. aureus* isolated from yak and cattle in Mongolia.

Introduction

Staphylococci are among the most significant pathogens causing a wide spectrum of diseases in both humans and animals. In humans, nosocomial and community-acquired infections are the most frequently reported (14, 30). Coagulase-positive (CPS) and coagulase-negative (CNS) staphylococci also are important mastitis pathogens in animals (1, 6, 7, 19, 20, 26, 31). Staphylococcus aureus is one of the most significant food-borne pathogens (16, 33). Raw milk and unpasteurised dairy products may contain enterotoxigenic strains of S. aureus, which may be associated with staphylococcal infections of the mammary gland (3, 8, 17, 21). Staphylococcus aureus can produce different exotoxins such as staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin 1 (TSST-1) (6, 7, 12, 13, 16, 24, 34).

So far, 20 serologically distinct SEs have been identified (18). However, the most common SE involved in S. aureus food poisoning is SEA (21). Different assays, such as tissue culture cell tests, immunoassays, reverse passive latex agglutination test and PCR techniques are widely used to detect SEs in food samples (2, 5, 14, 18, 21, 23, 25, 29, 32).

The ability of S. aureus strains to produce one or more SEs in food products is linked to staphylococcal food poisoning (SFP). It is characterised by an acute onset of nausea, vomiting, abdominal cramps and diarrhea. The symptoms occur when foods containing enterotoxin are ingested. The amount of SE capable of causing intoxication is uncertain, but there is an indication that an enterotoxin dose of less than 1.0 μg in contaminated food will cause symptoms of food poisoning (2, 9). Although not considered especially lethal, death can ensue if large amounts of SEs are ingested: fatality rates range from 0.03% in the general population to as high as 4.4% for highly sensitive persons such as immunocompromised persons, elderly persons and children (2).

Food poisoning is a universal public health concern. Therefore, it is important that foods and raw ingredients, including milk, are subject to microbiological controls. However, there are no reports on the occurrence of enterotoxigenic staphylococci in raw milk samples from Mongolia. Dairy products, including milk, cheese, cream, butter and yoghurt, are important and primary sources of nutrition in Mongolia. Mongolia is a pastoral country and 80% of milk is produced from free-grazing cattle and yak.

Thus, the aim of this study was to investigate the occurrence of and enterotoxin production by *S. aureus* isolated from raw milk from yak and cattle in Mongolia.

Materials and methods

Study area

The study region for the present work was an area of approximately 50 km^2 in the Sharhooloi and Bayan Dohom valleys, Gachuurt village, Mongolia. The area of Gachuurt village is a major milk-producing region near Ulaanbaatar city. Dairy cattle and yak were kept in open housing and milked twice daily.

Sampling

Between July and August 2004, 97 milk samples were taken: 65 milk samples were randomly taken from yak and 32 samples were obtained from cattle. The teat ends were cleansed with alcohol swabs and allowed to dry. The first stream was discarded and then 10 ml of milk was collected in 15 ml disposable sterile screw-cap tubes. Samples were immediately transported to the Veterinary Sanitation and Hygiene laboratory, Institute of Veterinary Medicine, Ulaanbaatar City, and kept at 4°C for no more than 24 h before freezing. From each sample, 1.5 ml of milk was pipetted into sterile microcentrifuge tubes and centrifuged at 10,000 rpm for 5 min at room temperature. The supernatant was then discarded and the pellet was stored at -20°C until laboratory processing. Samples were then transported with frozen cool packs to the Department of Biomedical Sciences and Veterinary Public Health, Faculty of Veterinary Medicine and Animal Science, SLU, Uppsala, Sweden.

Isolation and identification

For the isolation, 10 µl of milk sediment was streaked onto blood agar plates (5% v/v bovine erythrocytes in heart infusion agar, Difco) and incubated at 37°C for 24 h under aerobic culture conditions. A number of presumed staphylococcal colonies, that formed on the plates (creamy, greyish, white, or yellow colonies, 2-5 mm in diameter) were examined by Gram-staining. Isolates containing Gram-positive and catalase-positive cocci were further subjected to coagulase, maltose, mannitol, and DNase tests and further identified with the API Staph system (BioMerieux, Marcy l'Etoile, France). The identification of *S. aureus* isolates was confirmed with the Phadebact Staph Aureus Test (Boule Diagnostics AB, Sweden) and by PCR amplification of the nuc-gene (*4*). The haemolytic properties of isolates were tested on blood-agar plates (5% v/v bovine erythrocytes in heart infusion agar, Difco).

Detection of SEs

The determination of SEs was evaluated with reverse passive latex agglutination. SEA, SEB, SEC and SED in the culture fluid of each *S. aureus* strain was detected with the SET-RPLA kit (Oxoid, Basingstoke, Hampshire, England), following the manufacturer's instructions.

Results

Of the 97 raw milk samples, 72 (74%) proved positive for staphylococci, of which 69% (50/72) were from yak and 31% (22/72) from cattle. Of the samples containing staphylococci, *S. aureus* was detected in 14% (7/50) of the yak milk samples and in 68% (15/22) of the cettle milk samples. The properties of the *S. aureus* isolates are listed in Table 1. From three of the samples, more than one strain of *S. aureus* was obtained (Table 1).

The results of SE production are shown in Table 2. Staphylococcal enterotoxin C (SEC) was detected in 19% (5/26) of the investigated *S. aureus* strains. Three of the enterotoxigenic strains were from yak and two strains from cattle. None of the *S. aureus* strains tested produced SEA, SEB, or SED.

Discussion

Food-borne infections and food poisoning are of major concern, worldwide. *Staphylococcus aureus* is one of the most common food borne bacterial pathogens that cause food poisoning in humans when ingested via contaminated food, including dairy products. In a recent report, *S. aureus* was detected in 75% of 220 bovine bulk milk samples (11). Futhermore, several investigators have described prevalences of 20-38% of *S. aureus* in raw milk products in Norway (10, 11, 15). In one Swedish report, CPS were detected in 38% of raw goat cheeses (28).

In the present study, of the 97 raw milk samples investigated, 72 (74%) were positive for staphylococci, of which 69% (50/72) were from yak and 31% (22/72) from cattle. Of the samples containing staphylococci, *S. aureus* was detected in 14% (7/50) of the yak milk samples and in 68% (15/22) of the cow's milk samples. In addition, 19% of *S. aureus* isolated from raw milk from cattle and yak were enterotoxigenic. All enterotoxigenic isolates produced SEC; however, none of the *S. aureus* tested produced SEA, SEB, or SED.

Different investigators have reported that *S. aureus* isolated from dairy products of bovine and ovine origin are able to produce high levels of SEC and SED. Olson et al. (22) found that 15% of 157 *S. aureus* isolates from mastitic cattle were enterotoxigenic; whereas Kenny et al. (13) reported that 28.6% of bovine *S. aureus* were enterotoxin producers. Furthermore, Stephan et al. (27) reported that 54% of bovine mastitic milk isolates enterotoxigenic, and Normanno et al. (21) reported 55.9% entrotoxin producing *S. aureus* isolates from raw milk in Italy. In contrast, Danish investigators did not detect enterotoxins in 160 *S. aureus* strains isolated from milk samples from cows affected by bovine mastitis (1). Nor did Jörgensen et al. (11) in Norway detect SE (SEA-SED) in 75 *S. aureus* isolates from a farm with small-scale production of raw milk cheese. The different rates of enterotoxin production found in these reports could be explained by the different

techniques used in these studies, differences in the origin of the isolates or by geographical differences.

In conclusion, *S. aureus* was found in raw milk samples from yak and cattle in Mongolia and some of the investigated *S. aureus* strains produced enterotoxin. To the best of our knowledge, this is the first report on the occurrence of and enterotoxin production by *S. aureus* from raw milk in Mongolia. The results warrant further investigations to elucidate the public health significance of *S. aureus*, as well as other food-borne pathogens, in milk in Mongolia.

Acknowledgements

We thank Dr. Helle Unnerstad for critical comments and suggestions and Lise-Lotte Fernström for excellent technical assistance.

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Table 1. *Properties of* S.aureus *isolated from raw milk from yak and cattle in Mongolia*

Strain	Origin	Coagulase	DNase	Maltose	Mannitol	Nuc gene	Phadebact test	Haemolysis
8:1	Yak	+	+	+	+	+	+	ß
16:1	Yak	+	+	+	+	+	+	ß
49:1	Yak	+	+	+	+	+	+	ß
51:1	Yak	+	+	+	+	+	+	ß
58:1	Yak	+	+	+	+	+	+	–
59:1	Yak	+	+	+	+	+	+	–
65:1	Yak	+	+	+	+	+	+	ß
71:1	Cattle	+	+	+	–	+	+	ß
72:1	Cattle	+	+	+	–	+	+	ß
73:1	Cattle	+	+	+	+	+	+	ß
74:1	Cattle	+	+	+	+	+	+	ß
75:2	Cattle	+	+	+	+	+	+	ß
77:1	Cattle	+	+	+	+	+	+	ß
78:1	Cattle	+	+	+w*	+	+	+	–
80:2	Cattle	+	+	+	+	+	+	–
81:1	Cattle	+	+	+	+	+	+	ß
81:2	Cattle	+	+	+	+	+	+	ß
82:1	Cattle	+	+	+	+	+	+	ß
82:3	Cattle	+	+	+	+	+	+	ß
83:1	Cattle	+	+	+	+	+	+	ß
84:1	Cattle	+	+	+	+	+	+	ß
85:1	Cattle	+	+	+	+	+	+	ß
91:1	Cattle	+	+	+	+	+	+	ß
92:1	Cattle	+	+	+	–	+	+	ß
92:2	Cattle	+	+	+	+	+	+	ß
92:4:2	Cattle	+	+	+	+	+	+	ß

w * weak reaction

Table 2. *Occurrence of enterotoxins in* S. aureus *isolates from raw milk from yak and cattle in Mongolia.*

Origin of *S. aureus*	No. of strains	No. of enterotoxin-positive strains				%
		SEA	SEB	SEC	SED	
Yak	7			3		42.9
Cattle	19			2		10.5
Total	26			5		19.2

Research report II

Antimicrobial susceptibility of *Staphylococcus* spp. isolated from milk samples from yak and cattle in Mongolia

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Abstract

There is a lack of information on pathogens causing mastitis and their antimicrobial susceptibility in yak and cattle from Mongolia. The purpose of this study was to determine the minimal inhibitory concentrations (MICs) of 12 antimicrobial agents for 45 isolates of *Staphylococcus* spp. from yak and cattle. Broth microdilution was used for the susceptibility testing and because of high oxacillin MICs, all isolates were also subjected to oxacillin agar screening and PCR for the *mecA* gene. The nitrocefin test was used to determine β-lactamase production. The proportion of resistance to β-lactams, based on β-lactamase production, was high (37-84%). However, no *mecA* gene was detected. Resistance to erythromycin, clindamycin, chloramphenicol, tetracycline, trimethoprim and fusidic acid was recorded among isolates from both yak and cattle. Cephalotin resistance was found only among coagulase-negative staphylococci from yak.

1. Introduction

Identification of mastitis pathogens is important when selecting effective antimicrobial agents. Antimicrobial agents are a primary tool for controlling staphylococcal mastitis, as are susceptibility patterns to guide therapy decisions. Thus, information on antimicrobial susceptibility for bacterial species within a particular herd is important for the therapy outcome (Owens et al., 1997). *Staphylococcus aureus* is an important pathogen causing both subclinical and clinical mastitis. Resistance to antimicrobials, particularly to β-lactam antibiotics is a major problem in the treatment of *S. aureus*. Widespread use of β-lactam antibiotics has resulted in the emergence of resistant organisms (De Oliveira et al., 2000, Erskine et al., 2002; Gentillini et al., 2000; Pitkälä et al., 2004). Coagulase-negative staphylococci (CNS) are increasing in importance as a cause of bovine mastitis in many countries and in for example Argentina and Finland the proportion of resistance to penicillin among CNS has been reported to be 27% and 32% respectively (Gentilini et al., 2002; Pitkälä et al., 2004).

Methicillin-resistant *S. aureus* (MRSA) is a major cause of hospital-acquired infections worldwide. In many parts of the world, an endemic level of MRSA is reached and community-acquired MRSA infections are also increasing. These bacteria are often multi-resistant and a serious public-health concern (Boyce et al., 2005). There are several reports on methicillin-resistant staphylococci (MRS) in dairy cows and in companion animals (Lee, 2003; Loeffler et al., 2005; van Duijkeren et al., 2004). The *mecA* gene determines methicillin-resistance in staphylococci. The gene encodes the penicillin-binding protein 2a (PBP2a), which has a reduced affinity for all β-lactams, including the penicillinase-resistant (reviewed by Chambers, 1997).

Milk production is an important source of income in Mongolia. Cattle are the most important but also yak, camels, horses, reindeer, sheep and goats are kept as milk producing animals. Mastitis occurs in yak but less frequently than in cattle (estimated to be around 15%). Farmers in Mongolia can buy antibiotics over the counter at the pharmacy. Hence, the usage of antibiotics is impossible to monitor. However, veterinarians are often consulted for treatment of mastitis, as the health of production animals in Mongolia is very important, for both economic and traditional reasons. Veterinarians in the field recommend tylosin, penicillin and tetracycline as first choices for treatment of mastitis. Frequent milking, acupuncture and ice are used to reduce the inflammation. Often a cream with povidone iodine is used to massage the udder. To our knowledge there is no other study published on antimicrobial susceptibility of mastitic staphylocci from Mongolia.

The purpose of this study was therefore to determine antimicrobial susceptibility of staphylococci isolated from milk samples from yak and cattle in Mongolia.

2. Materials and methods

2.1. Source and identification of bacterial isolates

Altogether 45 isolates of staphylococci were used in the study. Tested isolates were from raw milk samples from yak and cattle in the Sharhooloi and Bayan Dohom valleys in Gachuurt village, Mongolia. The isolates were from cattle with subclinical mastitis while the yak isolates were taken from animals without any diagnosis of either acute or subclinical mastitis. The isolates were stored in brain heart infusion (BHI, Difco) broth with 17% glycerol at -70 °C. Isolates were characterized as *Staphylococcus*, based on colony morphology and hemolytic properties. Gram and catalase positive isolates were further subjected to coagulase, maltose, mannitol, DNase tests and PCR amplification of the *nuc*-gene (to be published elsewhere).

2.2. Susceptibility testing

The susceptibility of the isolates to antimicrobials was determined by broth microdilution (VetMIC GP-mo-A; National Veterinary Institute (SVA), Uppsala, Sweden). The standards from the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) were followed (NCCLS, 2002). Prior to the testing, the isolates were subcultured on blood agar and incubated for 16 h at 37 °C. Cation Adjusted Mueller-Hinton broth (CAMHB), (Difco) was used as a test medium and microdilution panels were incubated at 35 °C for 18 h. The MIC was read as the lowest concentration completely inhibiting visible growth. The oxacillin MIC was read a second time after 24 h incubation. Additionally, all isolates were screened for methicillin resistance by oxacillin agar screening (NCCLS, 2003). *Staphylococcus aureus* ATCC 15915, *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus aureus* CCUG 35601, *Staphylococcus epidermidis* ATCC 29886, *Staphylococcus epidermidis* ATCC 29887 were used as quality control strains.

2.3. β-lactamase production

The production of β-lactamase was tested by using nitrocefin discs (AB Biodisk, Solna, Sweden). The discs are impregnated with a chromogenic cephalosporin of which the enzyme ruptures the β-lactam ring, and this results in a colour change, from yellow to red. The tests were performed with the plate method, in accordance with the manufacturer's instructions.

2.4. Detection of *mecA*

The presence of the *mecA* gene was detected by PCR as described previously (Smyth et al., 2001). From the blood agar plate, 1 µl bacterial material was picked and suspended in 100 µl sterile water. The samples were boiled for 15 min, cell debris was removed by centrifugation and 2 µl of the supernatant was used as a template. The cycles used were 94 °C for 3 min for the first cycle; 94 °C for 10s

and 53 °C for 20s for the next 30 cycles, and 72 °C for 5 min in the last cycle. The amplicons were analysed by electrophoresis in a 1.5% agarose gel. The *mecA*-positive control organism included was *S. epidermidis* ATCC 29887.

3. Results

The MIC determination results and the percentage of resistance are summarized in Tables 1, 2 and 3. The oxacillin MICs read after 24 h of incubation are presented. The microbiological cut-off values for resistance used in the tables are from the Swedish Veterinary Antimicrobial Resistance Monitoring programme (SVARM, 2004), except for oxacillin (cut-off >4 µg/ml). These cut-off values are based on the distribution of MICs for *S. aureus* and for each antimicrobial tested. Isolates significantly deviating from the normal susceptible population are designated as resistant. No cut-off value was given for *S. aureus* and fusidic acid in the SVARM programme and in this study >0.5 µg/ml was used.

No isolate was resistant to more than three of the antimicrobial agents tested. All isolates tested were susceptible to gentamicin, neomycin and enrofloxacin. Of the *S. aureus* isolates from cattle, four were resistant to both erythromycin and clindamycin. Three of these were also resistant to chloramphenicol, as were another four isolates and one *S. aureus* from yak. The only isolate resistant to trimethoprim was a *S. aureus* from cattle.

Isolates of staphylococci from yak were mostly identified as CNS (73%) and they were more resistant to penicillin (84%) than the *S. aureus* isolates. High MICs of oxacillin (>4 µg/ml) were observed among the CNS isolates from yak and these isolates were also resistant in the oxacillin agar screening, yet all isolates in this study were negative in the *mecA* PCR (Table 4). One CNS isolate was resistant to both erythromycin and chloramphenicol. Resistance to cephalothin, oxytetracycline and fusidic acid was found only among the CNS.

In the nitrocefin test, 21 out of 26 isolates (81%) from yak and 7 out of 19 isolates (37%) from cattle were tested β-lactamase positive. Altogether 28 staphylococci isolates (62%) were positive for β-lactamase production.

4. Discussion

Four *S. aureus* isolates from cattle were resistant to both erythromycin and clindamycin. Cross resistance to macrolides, lincosamides and streptogramin B (MLS_B) is caused by *erm*-genes in *S. aureus*. Methylation of adenosine 2058 (*E. coli* numbering) in 23S rRNA causes reduced binding to the ribosome for these antimicrobial agents. The CNS isolate resistant to erythromycin only, could have inducible resistance to clindamycin, or the erythromycin resistance is caused by an efflux protein encoded by the *msrA* gene. If clindamycin is used for therapy for

such an isolate, the double disc agar diffusion test is recommended to detect possible inducible clindamycin resistance (Rich et al., 2005).

Of the *S. aureus* isolates, seven from cattle and one from yak, together with one of the CNS isolates were resistant to chloramphenicol. Resistance to chloramphenicol in staphylococci is mediated by enzymes, chloramphenicol acetyltransferases (CATs), inactivating the substance (reviewed by Schwarz et al., 2004). Recently a new resistance mechanism for *Staphylococcus* spp. was described, the *cfr*-gene encoding for a protein that methylates the adenosine 2503 in 23S rRNA, causing resistance to both chloramphenicol and clindamycin (Kehrenberg et al., 2005). Earlier, chloramphenicol has been used to treat cattle and yak in Mongolia for diseases other than mastitis. Today, to our knowledge, veterinarians in Mongolia do not prescribe chloramphenicol for animals.

The CNS were more resistant to β-lactams than *S. aureus* which is contradictory to other studies (Gentilini et al., 2000, 2002; Pitkälä, et al., 2004). One difference is that all the CNS isolates in this study were from yak. The *S. aureus* from yak had an equally high proportion of β-lactam resistance, though only seven isolates were included.

Because of the high MICs of oxacillin (>4 µg/ml) for the CNS isolates, oxacillin agar screening and a PCR for the *mecA* gene were performed (Table 4). Methicillin resistant staphylococci have been found in milk from cattle (Lee, 2003). However in the present study no *mecA* positive isolates were detected. The high MICs of oxacillin can be explained by other resistance mechanisms such as overproduction of β-lactamase, over expression of PBPs, or modification of PBPs (Chambers, 1997).

Cephalothin was included to test for resistance against all first-generation cephalosporins. Resistance was found only among the CNS isolates. The cut-off value for resistance used is based on the MIC distribution for *S. aureus* and is not optimal for the MIC distribution for CNS. The CNS group is heterogeneous and the material in this study limited. Most likely, different cut-off values would be appropriate for *S. aureus* and CNS and also for different CNS species. Hence, the cephalothin resistance percentage should be interpreted with caution.

A recent study from Finland shows that the nitrocefin test agreed better with the presence of the β-lactamase gene (*blaZ*) detected by PCR than did the penicillin MICs obtained by agar dilution (Haveri et al., 2005). In this study we present the penicillin resistance as the percentage positive in the nitrocefin test instead of using a MIC cut-off value (Tables 1-3).

Because of the limited number of isolates investigated, it is difficult to draw firm conclusions. Nevertheless, it can be concluded that resistance to β-lactams, erythromycin, clindamycin, chloramphenicol, tetracycline, trimethoprim and fusidic acid does occur among staphylococci from yak and cattle in Mongolia. Furthermore, resistance to first-generation cephalosporins was recorded for CNS isolates. For the isolates in this study the frequency of resistance to β-lactams was high, but no MRS were found.

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Table 1. *Distribution of MICs of 12 antimicrobial agents for 19* S. aureus *isolates from cattle.*

Substance	Resistance (%)	Distribution (No. of isolates) of MICs[1] (mg/L)												
		≤0.03	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
Penicillin	37[2]	3	5	4			1			6					
Cephalothin	0				2	13	4								
Oxacillin	0					1	3	10	5						
Erythromycin	21					14	1						4		
Chloramphenicol	37									11	1		7		
Clindamycin	21				14	1							4		
Oxytetracycline	0					4	15								
Fusidic acid	0				15	4									
Gentamicin	0					12	7								
Neomycin	0						18	1							
Enrofloxacin	0			1	8	10									
Trimethoprim	5						1	8	9				1		

[1] White fields denote range of dilutions tested for each substance. Bold vertical lines indicate breakpoint for each substance. MICs above the range are given as the concentration closest to the range. MICs equal to or lower than the lowest concentration tested are given as the lowest tested concentration. [2] Based on β-lactamase production.

Table 2. *Distribution of MICs of 12 antimicrobial agents for 7* S. aureus *isolates from yak.*

Substance	**Resistance** (%)	**Distribution of MICs** (mg/L)												
		≤0.03	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
Penicillin	71[2]	1	1						1	4					
Cephalothin	0				1	4	2								
Oxacillin	28.6					1	1	3	2						
Erythromycin	0				1	6									
Chloramphenicol	14.3									6			1		
Clindamycin	0				7										
Oxytetracycline	0					5	2								
Fusidic acid	0				1	6									
Gentamicin	0					5	2								
Neomycin	0						7								
Enrofloxacin	0			2	5										
Trimethoprim	0							6	1						

[1] White fields denote range of dilutions tested for each substance. Bold vertical lines indicate breakpoint for each substance. MICs above the range are given as the concentration closest to the range. MICs equal to or lower than the lowest concentration tested are given as the lowest tested concentration. [2] Based on β-lactamase production.

Table 3. *Distribution of MICs of 12 antimicrobial agents for 19 CNS isolates from yak.*

Substance	**Resistance** (%)	**Distribution of MICs** (mg/L)												
		≤0.03	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**
Penicillin	84[2]		4		1	2	4	2	2	4					
Cephalothin	53			3	3		3	4	4		2				
Oxacillin	58						1	4	3		1	10			
Erythromycin	5				16	2						1			
Chloramphenicol	5								14	2	2		1		
Clindamycin	0				6	6	4	3							
Oxytetracycline	10					13				4				2	
Fusidic acid	21				11	4			2	2					
Gentamicin	0					19									
Neomycin	0						19								
Enrofloxacin	0				12	7									
Trimethoprim	0							4	15						

[1] White fields denote range of dilutions tested for each substance. Bold vertical lines indicate breakpoint for each substance. MICs above the range are given as the concentration closest to the range. MICs equal to or lower than the lowest concentration tested are given as the lowest tested concentration. [2] Based on β-lactamase production.

Table 4. *Oxacillin susceptibility test results obtained for* S. aureus *isolates from yak and cattle and CNS isolates from yak.*

Isolates and strains	Origin	Oxacillin MIC[1] (µg/ml)	Oxacillin agar screening
S. aureus			
8:1	Yak	2	S
16:1	Yak	2	S
49:1	Yak	4	S
51:1	Yak	2	S
58:1	Yak	1	S
59:1	Yak	0.5	S
65:1	Yak	4	S
71:1	Cattle	2	S
72:1	Cattle	2	S
73:1	Cattle	2	S
74:1	Cattle	2	S
75:2	Cattle	2	S
77:1	Cattle	1	S
78:1	Cattle	4	S
80:2	Cattle	4	S
81:1	Cattle	4	S
81:2	Cattle	2	S
82:1	Cattle	1	S
82:3	Cattle	1	S
83:1	Cattle	2	S
84:1	Cattle	4	S
85:1	Cattle	0.5	S
91:1	Cattle	2	S
92:1	Cattle	2	S
92:2	Cattle	4	S
92:4:2	Cattle	2	S
CNS			
5:1	Yak	2	S
10:1	Yak	>16	R
13:2	Yak	>16	R
15:1	Yak	4	S
17:2	Yak	>16	R
20:1	Yak	>16	R
21:1	Yak	>16	R
22:1	Yak	>16	R
22:2	Yak	>16	R
23:1	Yak	>16	R
24:1	Yak	>16	R
24.2	Yak	>16	R
24:3	Yak	4	S
25:2	Yak	1	S
28:1	Yak	>16	R
29:2	Yak	>16	R
30:1	Yak	2	S
50:2	Yak	2	S
52:2	Yak	2	S
Control strains			
S. aureus ATCC 15915		2	S
S. epidermidis ATCC 12228		1	S
S. aureus CCUG 35601		>16	R
S. epidermidis ATCC 29886		1	S
S. epidermidis ATCC 29887		8	R

[1]2% NaCl added to the broth

Acknowledgements

This study was carried out at the Department of Biomedical Sciences and Veterinary Public Health, Faculty of Veterinary Medicine and Animal Science, SLU. I am grateful to the Swedish Foundation for International Co-operation in Research and Higher Education (STINT) for providing me with a scholarship, which enabled me to take my Master's degree.

I would like to express my greatest gratitude to the following people who directly or indirectly contributed for the completion of this study:

Prof. Karin Östensson, Marie Sundberg and all the staff (SIPAR) for my acceptation to the special Master of Science Programme in Veterinary Medicine for International Students;

Prof. Martin Wierup, Head of the Department of Biomedical Sciences and Veterinary Public Health, for welcoming to the department. Thank you for your great support and encouragement;

Dr. Karel Krovacek, my supervisor, for affording me the opportunity to participate in the proposed Master's project and for sharing your great knowledge in the field of microbiology. Thank you for your valuable suggestions, guidance to the laboratory work and all help in preparing my manuscript;

Dr. Märit Pringle, my co-supervisor, for teaching me the molecular techniques, including both practical and theoretical issues. Thank you for valuable suggestions and all help in preparing my manuscript;

Dr. Helle Unnerstad, my co-supervisor, for kindness and valuable comments and suggestions during preparation of my thesis;

Dr. Giovanni Normanno, for fruitful scientific collaboration;

Margareta Horn af Rantzien, for the assistance and guidance in the experimental work with the antibiotic panels, and the fun.

I am grateful to all the staff at the Division of Bacteriology and Food Hygiene: Olov Carlsson, Lise-Lotte Fernström, Mona Fredriksson, Karin Hendelberg, Fredrika Ingermaa, Karl-Erik Johansson, Anne-Sofie Lundquist, Britt-Marie Thorberg, Gunilla Trowald-Wigh, for their warm and friendly help. I am especially grateful to Helena Höök for her kind help, comments and suggestions during the final correction of my thesis.

My special thanks to Anna Birgersson and her lovely family.

To Mr. Max Brandt for last-minute linguistic editing of the manuscript, comments and suggestions.

I am grateful to all the staff at the Institute of Veterinary Medicine, Ulaanbaatar, Mongolia and especially Prof. N.Erdenetsogt, Dr. B.Byambaa for giving me the opportunity to set out on my journey of scientific work. I am grateful to B.Enkhelmaa for collaboration in the laboratory and providing the samples.

To my friends, international Master's students, Pushkar Kulkarni, Inoka Peiris, Iftikhar Ali, Charles James Ley, Anas Al-Makhzoomi, Sowsan Taha for true friendship and support.

I am deeply grateful to my parents and sisters for their endless love and support. Thanks to my loving family.

Thanks to Lord Buddha.